



Using image analysis for counting larvae of potato cyst nematodes (*Globodera* spp.)

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Summary – A GOP-302 image analysis system (Context Vision, Sweden) was used to automate counting large numbers of *Globodera rostochiensis* and *G. pallida* larvae in suspension. These suspensions originated from hatching tests, which were conducted to estimate percentage mortality of nematodes exposed to nematicides in field and lab experiments. The result is called ANECS (Automatic Nematode Counting System), a software program that can count up to 64 compartments with larvae suspensions successively without the aid of an operator. A special object carrier was developed. Images of up to eight object carriers (512 larvae suspensions) can be stored and image analysis can be performed during off-office hours. The time needed to count one compartment was reduced by 80 % to one minute compared to “ manual ” labour, while the time for probe preparation remained the same. The percentile error is highest at very low larvae densities (< 20 per suspension) and is caused by contamination with small fibres carried by the air during the handling of the larvae suspensions. This problem can be minimized by establishing clean laboratory procedures. At least 95 % of the larvae originating from hatching tests were recognized and counted. The program can, and has been, adapted for counting other nematode species or to suit more complicated problems like counting both larvae and eggs in one suspension.

Résumé – Utilisation de l'analyse d'image pour le comptage des larves des nématodes à kystes de la pomme de terre (*Globodera* spp.) – Le système GOP302 d'analyse d'images (Context Vision, Suède) a été utilisé pour automatiser le comptage de nombres élevés de larves de *Globodera rostochiensis* et *G. pallida* en suspension. Ces suspensions provenaient de tests d'éclosion menés pour estimer le pourcentage de mortalité lors d'expériences aux champs et en laboratoire sur des nématodes exposés aux nématicides. Le résultat, appelé ANECS (Automatic Nematode Counting System), consiste en un logiciel programmé pour compter successivement jusqu'à 64 compartiments contenant ces suspensions, et ce sans l'aide d'aucun opérateur. Un porte-objet adapté a été mis au point. Les images provenant de huit porte-objet (512 suspensions de larves) peuvent être stockées et leur analyse différée en fonction des heures de travail. Le temps nécessaire au comptage d'un compartiment est d'une minute, correspondant ainsi à une réduction de 80 % du temps d'un comptage « manuel », tandis que le temps de préparation de l'échantillon reste inchangé. L'erreur percentile est la plus élevée dans le cas de densités faibles de larves (< 20 par suspension); elle est causée par la pollution provenant de petites fibres transportées par l'atmosphère durant la préparation des suspensions de larves. Ce problème peut être diminué en adoptant la procédure dite des « laboratoires propres ». Au moins 95 % des larves provenant des tests d'éclosion sont reconnues et comptées. Ce programme peut être, et a été, adapté au comptage d'autres espèces de nématodes ou même à des problèmes plus compliqués comme le comptage des œufs et des larves dans la même suspension.

Key-words : automated counting, *Globodera* spp., image analysis, nematodes.

One of the most laborious and tedious occupations in nematological research is the counting of individuals of a pathogen, for instance, the number of cysts in debris, extracted from a soil sample, or the number of eggs and/or larvae in a suspension. From 1985 to 1990, a substantial part of the research effort at the nematological department of IPO-DLO was directed towards the effectiveness of nematicides in the control of potato cyst nematodes. Dosage-response relationships of 1,3-dichloropropene (D-D) (Schomaker & Been, 1986) and methylisothiocyanate (MIT) were investigated in laboratory tests, and the effectiveness of D-D was tested

on fields with heavy marine clay soil (Been & Schomaker, 1987). In all these experiments, percentage mortality of potato cyst nematodes was estimated.

Several methods for distinguishing between living and dead potato cyst nematodes are available, ranging from “pricking” individual eggs (Fielding, 1951), the “kinked larvae” method (Staniland & Stone, 1953), bioassays (Hijink, 1971), colouring with various dyes, e.g., new blue R (Shepherd, 1951), hatching tests (Fenwick, 1949, 1950), and measuring the adenosine triphosphate (ATP) content of nematodes (Atkinson & Ballantyne, 1977a, b). The last three methods are fre-

quently used. Bunt and van Eck (1978) compared these methods, including a bioassay and a measurement of mortality of free living nematodes to estimate mortality after a soil fumigation, and found the hatching test to be one of the most reliable methods. Therefore, in all the experiments, percentage mortality was estimated by comparing the hatching of treated larvae with that of untreated larvae.

The efficiency and reliability of hatching tests were increased by several methodological improvements which will be presented in another paper. The duration of the hatching tests varied from two weeks with untreated nematodes to six months with nematodes exposed to high dosages of nematicides (Schomaker & Been, 1986). A similar retardation of hatch, caused by nematicides was reported earlier (Fenwick, 1957; Seinhorst & Den Ouden, 1973). Every week, the hatching solution had to be refreshed and hatched larvae counted to plot the sigmoidal hatching curve (Fenwick, 1950). The many different factors involved (fields, plots, soil layers, time after treatment, and replicates) resulted in a considerable number of batches and thousands of larvae suspensions to be counted. "Manually" counting one suspension takes five minutes in average, and only few people can perform this tedious microscopy work during a whole day. Moreover, the reliability of the manual counts decreases as the duration of this work increases.

The possibility to automate this task was investigated using the (at this time) relatively new method of computerized image analysis of digitized images. Several large systems were pre-tested in 1986/1987 for processing speed, image analysis software, and user friendliness. Finally, GOP-302 (from Context Vision, Sweden) was selected and equipped with a suitable selection of peripheral instruments. This paper presents the results of three-year research effort into the composition of a suitable microscope configuration and the development of the required software.

Materials and methods

THE SYSTEM'S HARDWARE CONFIGURATION

A GOP-302 image analysis system, manufactured by Context Vision, Sweden, was used to automatically estimate the number of nematode larvae (*Globodera rostochiensis* and *G. pallida*) in suspensions. An extra hard disk of 700 MB was added for the storage of large numbers of images. A Leitz Stabiplan microscope was selected as the most suitable for this application. It is an industrial microscope for automated applications, consisting of a solid granite base to obtain maximum stability during the scanning stage movements and a system adapter for a Wild M420 Macroscop or a binocular tube for micro objectives. For our purpose, a binocular tube was used. The center of the system adapter extends 26 cm, enough to equip it with a large scanning stage. A photo-tube is attached on top of the binocular tube (1.25 ×

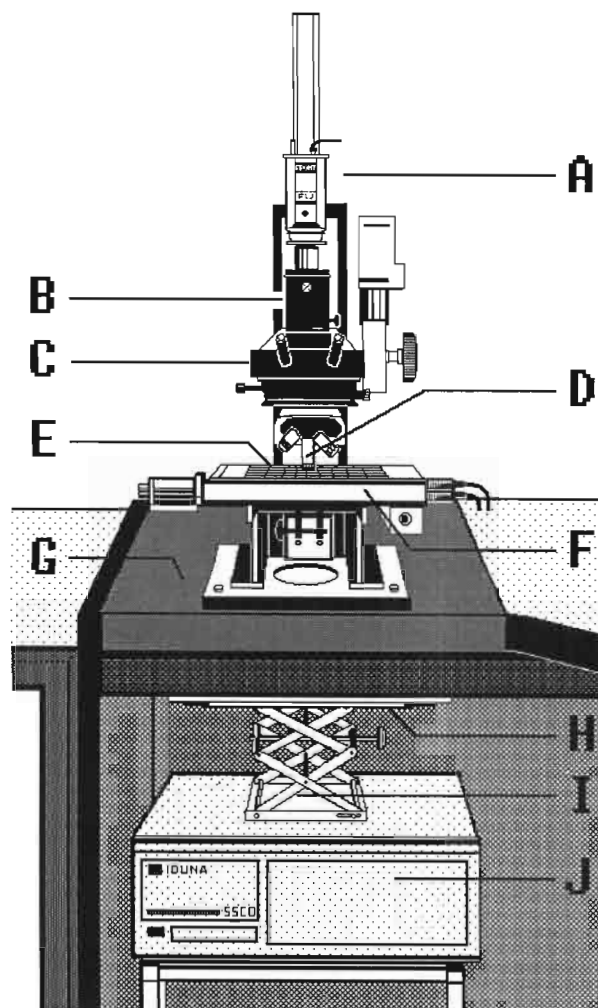


Fig. 1. Overview of the microscope setup. A: 2/3" B/W CCD camera; B: 0.4 × photo tube; C: Leitz Stabiplan microscope; D: 1.6/0.05 P objective Leitz; E: multiple compartments object carrier; F: 8" Scan stage; G: Granite base; H: Electronically controlled light source; I: Labo lift; J: Stage and focus controller.

with a projection lens (4 ×) which is connected to the camera with a C-mount adapter (0.1 ×). See Fig. 1 for a display of the microscope set-up. In combination with a Leitz 1.6/0.05 PLAN objective, the total magnification obtained was sufficient to display an image frame on the monitor covering exactly 1/4 of the total area of one compartment. The lens combination used was the best compromise between a large magnification needed for sufficient contrast and a small magnification to obtain a large scanning area. A 8" Märzhäuser scanning stage was used, with a maximum speed of 42 mm/s and an accuracy of 2.5 mm. It is mounted to position the perspex 64 compartment object carriers specially developed for this purpose by Hasselblad, Sweden. The scanning stage (SSCO2) is controlled by the computer using a

stage and focus controller developed by IDUNA, The Netherlands. For optimal illumination of the objects, an electronically stabilized Novalux A4EIR light source was used, emitting homogeneous light in an A4 area.

OBJECT CARRIER

A special object carrier with 64 compartments had to be developed fitting the 8" Märzhäuser scanning stage. Several prototypes of different construction were developed and tested. The first consisted of one layer of plastic (PVC). The object compartments were formed by vacuum suction of the material after heating. In this way compartments had slanted sidewalls, resulting in no visible connections of larvae to the walls of the compartment after segmentation. However, during the cooling period following manufacturing, the object carriers slightly deformed, making them not completely level with the surface of the scanning stage. As a result the bottom of the compartments drifted out of focus during movement of the scanning stage. Auto focusing had to be added to solve this problem but scanning time was increased considerably, as each final image of a compartment was the result of images of all four quadrants of the compartment. Moreover, auto focusing at this low magnification tended to fail.

A second prototype, also with slanted walls, consisted of two perspex layers glued together, the lower one solid, the upper one with compartments cut out by laser. These object carriers were level by definition, therefore auto focusing became unnecessary. However, after gluing, the connection of the slanted compartment wall with the bottom perspex plate produced clearly visible lines in the scanned images. Image analysis also suffered from glue stains on the bottom of the compartments. Both artifacts tended to connect with larvae during segmentation, causing serious problems with the recognition of these larvae. Moreover, the glue slowly dissolved after the killing/staining solution was added to the larvae suspensions. After a few runs, the object carriers started leaking between plates and were useless.

The final development (Fig. 2) also consisted of two perspex plates. A new glue was used which was sprayed over the bottom of the upper perspex plate, afterwards, both plates were combined by heat and pressure. Glue artifacts were almost absent. To enable straight-forward segmentation, the object compartments now had vertical walls and the upper surface of the multiple compartment object carrier was painted black to obtain maximum contrast with the bottom plate. During image analysis, the black edge of the upper perspex plate around the compartment can be subtracted from the image and touching larvae dissociated easily. Each compartment is 15×16 mm, and has a volume of approximately 1.2 ml. The whole object carrier measures 24.5×24.5 cm.

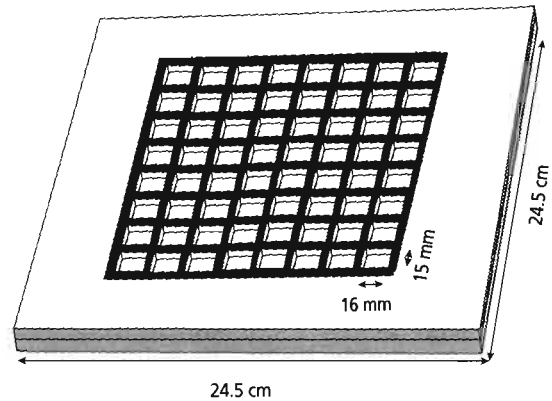


Fig. 2. Object carrier with 64 compartments. The whole object carrier measures 24.5×24.5 cm. The compartments are centered on the carrier in a 8×8 square of 15.6×15.6 cm. Each compartment measures 15×16 mm and has a volume of approximately 1.2 ml.

BIOLOGICAL SPECIMENS

Cysts of *Globodera rostochiensis* and *G. pallida* were soaked in water and crushed in suspension using a plunger (Seinhorst & Den Ouden, 1966). Eggs were retrieved by sieving out cyst walls with a 250 µm sieve. Eggs were counted in a 1 ml suspension (Seinhorst & Den Ouden, 1973) to estimate those present in the stock solution. A volume containing the desired number of eggs was pipetted into a glass tube (1000 eggs for untreated batches) sealed at the bottom by a gauze (22.4 mm, Monadur), which retains eggs but not active larvae. The tubes were then placed into glass cups containing 1 ml of the natural hatching agent of potato plants. When necessary, tubes were placed in new cups containing fresh hatching agent. Cups containing hatched larvae were stored in the refrigerator at 4 °C. Storage time never exceeded 4 weeks as after such a period of time the food resources of the nematodes decrease and starch globules diminish in size and finally become completely absent. This makes staining of the larvae for image analysis impossible, and the recognition of objects unreliable. The whole cup, containing slightly less than 1 ml of larvae suspension, was emptied and rinsed into the selected object compartment. Before counting, 20 ml of a saturated iodine solution (iodine dissolved in 96 % alcohol) was added, which caused larvae to die instantaneously, stretch and stain. If necessary, a small amount of bidistilled water was added to obtain a level surface of the fluid. Image acquisition (scanning) was performed after a pause of 5 min, in order for the larvae to gain maximal contrast, but within 1 hour as the larvae lose their stain after that period. Moreover, waiting for too long causes evaporation of the sample fluid resulting in a meniscus that blurs the image.

Larvae from these hatching tests were used to investigate which features, e.g., length, area, compactness, can be used to distinguish them from other objects by image analysis algorithms. After program building, hatching tests were analyzed both by the automated system and by manual counting to establish the efficiency and reliability of the developed algorithm.

Results

DISCRIMINATING FEATURES

Segmentation of objects from a background can be achieved by thresholding a grey value image, which consists of a number of picture elements (pixels), each having a grey value between 0 and 255. When thresholding the image, all pixels with a grey value beneath a certain threshold value ($0 \leq \text{value} \leq 255$) will be adjusted to zero: all others will be adjusted to one. Thus, a binary image is created consisting of a background (value = zero) with one or more objects, consisting of one or more pixels with the value one. These objects can be submitted to calculations. One of the simplest calculations is counting the number of pixels that form an object as a measure of its area (see Rosenfeld and Kak, 1976 for a general introduction in image analysis). The GOP-302 can measure up to seventeen different features of each object almost instantaneously. Using the values obtained for a number of chosen features of the object, decisions can be made whether these values are within the accepted range for each feature and therefore, whether the object can be classified as a larvae or not (e.g., egg or artifact caused by contamination of the suspension). Therefore, the first step in building the algorithm is to investigate which of the features or combination of features allow reliable discrimination between larvae and other objects.

As suspensions can contain several hundred larvae per ml, many larvae will coalesce into clusters after thresholding, thus forming a single object. Therefore, the algorithm also has to distinguish between single larvae, double larvae, and clusters of more than two larvae.

A combination of three features proved to be the most suitable to achieve the desired purpose. For single larvae: **area** is used to find all objects having an area which falls within a range of area measurements likely to originate from a single larva. **Compactness** is employed as a qualifier to remove all objects which have the area of a single larva but not its shape. **Compactness** is a measurement of shape; circular objects will get the value 1, while the more unlikely object resembles a circle, the higher the value for **compactness** will be. Finally **ellipse compactness**, also a shape measurement, is applied as a third qualifier. See GOP-302 reference manual (Anon., 1987) for an explanation of the quoted features.

For pairs of larvae and clusters, the same approach is used with adapted values of the different features. In

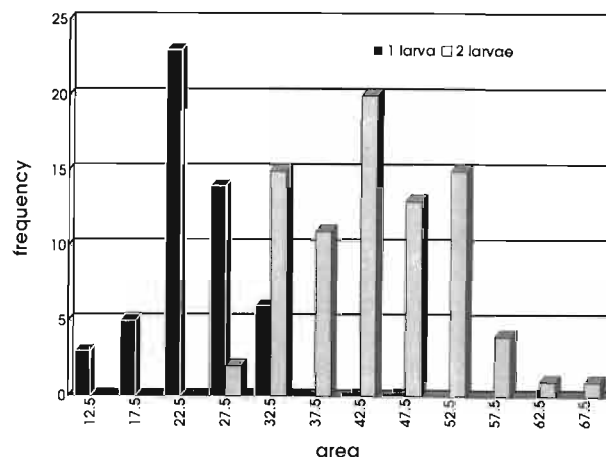


Fig. 3. Frequency distribution of **area** of single larvae and clusters of two larvae. **Area** is used as a classification feature. Although a slight overlap does occur, the resulting error is negligible.

Fig. 3, the frequency distribution of several **area** measurements are displayed for single and paired larvae. A slight overlap occurs but the resulting error is negligible as two more qualifiers are used for the final decision. After classification a C-program is invoked to calculate the average area of a single larva using the **area** measurements of all recognized single and double larvae. The number of larvae in clusters is then calculated by dividing the cluster area by the average single nematode area. This procedure is repeated for every compartment. For a full description of the algorithm used, see the ANECS user manual (Been *et al.*, 1995).

PROGRAM PERFORMANCE

After program development, debugging, and evolution, the performance of the final program was tested by applying it to several samples of nematode larvae originating from various hatching tests. In Fig. 4, automated counts are expressed as percentages of manual counts. There seems to be a tendency to overestimate the number of larvae at low numbers, especially those below 20 larvae per suspension. Overestimation is caused by pollution of the larvae suspension. As the image enhancement algorithm (see Been *et al.*, 1995) takes care of most objects that do not have the shape of a larva, only those that resemble larvae cause errors. The contamination of the larvae suspension, mostly by small fiber particles originating from clothing, is carried by air and takes place when vials are handled and the compartments of the object carrier are loaded. Clean-laboratory procedures and air handling could drastically reduce these errors.

At higher nematode numbers, at least 90 % of the larvae are detected as such. The underestimation was caused by a classification error in the algorithm. When suspensions with very high nematode numbers were analyzed, large clusters of larvae can be formed. These

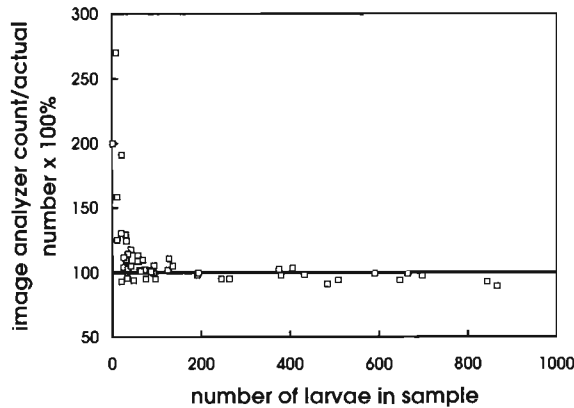


Fig. 4. Percentile count obtained by computer analyses compared to the actual number of larvae present in the sample. The highest errors occur with low nematode numbers (< 20). They mostly are the result of small contaminations in the liquid (e.g., tissue fibers transported by air).

were sometimes ignored by the program as they were classified as a large object (such as a piece of cyst shell) polluting the suspension. Moreover, clusters with an area of more than 50 single larvae are automatically rejected as these objects are regarded as contamination by definition. This rule was implemented as a consequence of results of previous testings.

However, a large percentile error at low numbers of nematodes causes no large errors at the end of a hatching test provided that suspensions with larger numbers of larvae are also counted, as is the case with hatching tests. Fig. 5 displays three examples of complete hatching tests with variable cumulative numbers of larvae at the end of the test, where automated counts are compared with manual counts. The smaller the final number of larvae, the smaller the errors made by the program. However, in all tests, at least 95 % of the larvae present were detected as such. The increasing error in nematode counts at higher total cumulative counts in Fig. 5 is again caused by the presence of very large clusters of larvae which were classified as contamination of the suspension and not as nematodes. At higher larvae numbers these clusters will occur more frequently.

Although a minimum of 95 % of the hatched larvae were detected as such, an attempt was made to solve the problem of unwanted rejection of large nematode clusters. Eventually, the use of an **area/length** index for clusters proved to be very promising. Fig. 6 demonstrates the linear relationship between area and length of larvae clusters. The calculated index is almost the same for any number of nematodes. However, large contamination with an area above 100 pixels can be detected because this feature would then be different. Rejection of these cluster having an **area/length** index above 2.3 and below 1.4, and acceptance of those within this

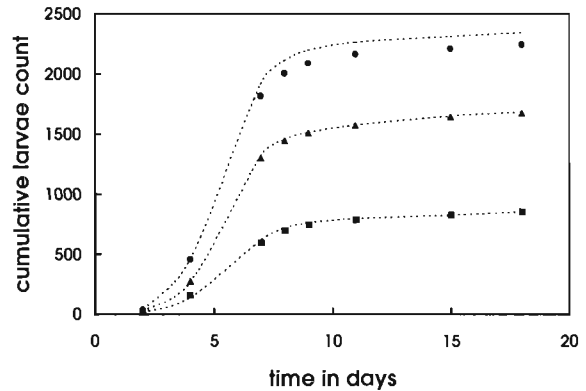


Fig. 5. Comparison between manual count and computer analysis of three hatching tests with different numbers of eggs. Lines : cumulative manual counts. Dots : cumulative counts of image analysis system. (■) 99 % of larvae detected, (▲) : 97 % of larvae detected, (●) : 95 % of larvae detected.

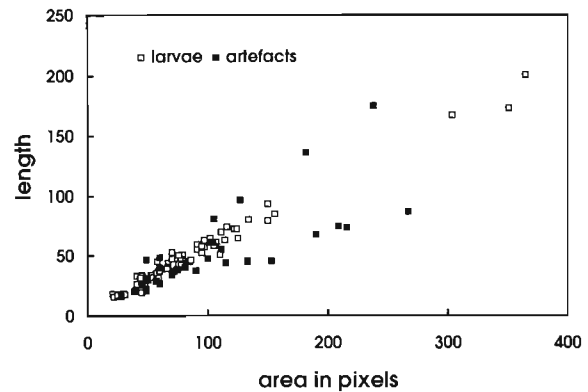


Fig. 6. Relation between **area** and **length** of clusters of more than two larvae; clusters of larvae (□) and contamination in suspension (■).

range, can decrease the number of erroneous rejections. Therefore, this index will be incorporated in the next update of the program.

Three batches of *G. pallida* and two of *G. rostochiensis* were investigated with regard to differences in size expressed as **area**, which is the most important classification feature of the program. As all populations were multiplied in previous years on the susceptible cultivar Irene, the differences between batches from each species were age differences : Ro1'86, Ro1'88, Pa3'84, Pa3'86, and Pa3'88. ANOVA distinguished two groups. One consisting of Ro1'86, Ro1'88 and Pa3'84 and the other consisting of Pa3'86 and Pa3'88 with an average area of 24 and 26 pixels, respectively. Frequency distributions of single batches were consistent with a normal distribution. Fig. 7 illustrates the frequency distribution of area measurements of both groups. Although there is a significant difference between the average **area** of both

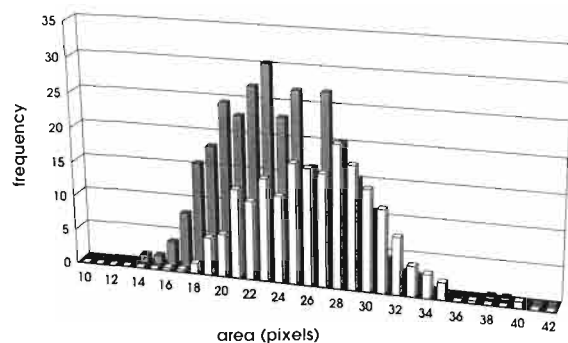


Fig. 7. Area measurements of single larvae of two different groups of nematodes originating from three batches of *Globodera pallida* and two batches of *G. rostochiensis*. Group 1 (grey blocks), consists of Ro1'86, Ro1'88, and Pa3'84. Group 2 (white blocks), of Pa3'86 and Pa3'88.

groups, both 95 % probability intervals (16 to 32 for group one and 18 to 34 pixels for group two) are within the range of the classification interval of **area** for one larva (10 to 35 pixels) used by ANECS. Therefore, the differences in **area** between the two groups have no influence on the performance of the program. It can be assumed that the program will perform equally well when other batches of potato cyst nematodes are counted. This has been proven during several years of operation.

Discussion

The result of this research has been a software application that can count up to 64 compartments with nematode suspensions without the aid of an operator. As scanning of the compartments is separated from the analysing part of the program, analysis of images (counting the larvae) can be effected during night hours to save operation time of the system during the day. After analysis, ANECS provides the possibility to review the results by displaying the original image together with a coloured overlay representing the detected and counted larvae (Fig. 8). Corrections can be made if very high precision is needed. For a full description of the program options see the ANECS user manual (Been *et al.*, 1995).

Special attention was paid to the implementation of laboratory procedures to obtain clean and non contaminated larvae suspensions. Best results were obtained when hatching cups were machine-washed, dried by filtered air, and stored in air tight boxes. Cups with suspension waiting to be counted were stored in plastic boxes at 4 °C. As the kitchen equipment needed for washing and drying is standard in modern laboratories, these precautions do not take extra time. The time required to prepare the probes is the same or even less, as 64 probes are prepared successively.

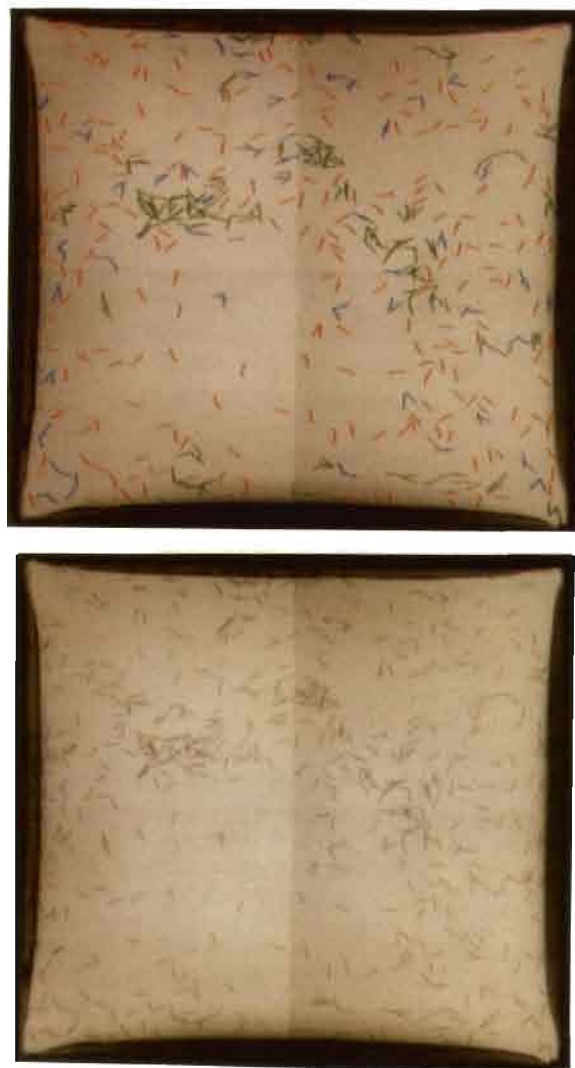


Fig. 8. Above : The original image after a C-subroutine called grab.c took four pictures of each quadrant of the selected compartment, reduced these images in size and composed one new image presenting the whole compartment; Below : Original image with coloured overlays displaying all objects which have been recognized as nematode larvae (single larvae in red, pairs in blue and clusters in green). The total sum of counted larvae is displayed on the top line of the image (not displayed here). Compartment number, number of single larvae, pairs, and clusters, and total number are stored in a data file.

As displayed in Fig. 4 the analysing process still suffers from errors caused by small amounts of contamination by fibers. Improvements could be made by avoiding drafts and using fiber-filtrated air and laboratory clothing at the location of the image analysis system. However, as can be concluded from Fig. 5, the error made by the program at the end of a hatching tests with several cumulative counts is low. At least 95 % of all

larvae of each sample were recognized and counted. This percentage can still be improved by adding the length/area index to the calculating algorithm of the program.

Whereas the time required for the manual count of one suspension averages 5 minutes, the image analysis system only requires an average of 1 minute for the same job, an 80 % reduction of counting time. Running the image analysis during night hours saves even more time. Another improvement is the absence of bias occurring when counts are made by different individuals and of the increasing number of errors caused by fatigue when an individual must perform this task for long periods.

The program has now been in operation for several years and satisfies the set goals. The program can be, and has been, adapted for counting other nematode species. For instance, to count beet cyst nematode larvae (*Heterodera schachtii*), only the upper and lower margins of the discriminating features used in the classification process had to be adapted. Larvae and eggs of *Meloidogyne hapla* and *M. chitwoodi* have been counted by Van der Beek *et al.* (1996). Adding egg recognition to the program proved to be quite simple. A filter operation is used to erase all thin objects, such as larvae, but not the eggs. Thresholding this image and classifying these objects renders a fair approximation of egg numbers.

The program is not suited to distinguishing between different species and therefore counting the numbers of different species in one suspension. This kind of image analysis would require texture analysis at very high magnifications, which implies constant autofocusing and movement of the scan stage as well as the use of stored recognition patterns to identify objects. This is theoretically possible for those species which do not have to be manipulated under the microscope to distinguish certain characteristics. However, even at the present state of the art, computers would consume too much time to equal, or even improve on, manual labour.

However, image analysis will prove to be of increasing value for nematological research. Heinicke and Schultz (1994) recently succeeded in solving another labor-intensive task: counting eggs and larvae in unfiltered suspensions of crushed cysts of *Globodera rostochiensis* and *Heterodera schachtii* originating from soil samples. Their method is intended for routine estimation of population densities in farmers' fields as a requisite in nematode control. The time required varies from 1 to 10 min per probe, depending on nematode species and number of nematodes in the suspension. The counting process is terminated when the variance is stable. Another possibility of using image analysis is demonstrated by Hendriks *et al.* (1994) who used the GOP-302 system to measure the lipid concentration in larvae of potato cyst nematode.

The GOP-302 image analysis system is now outdated. ANECS is currently converted to run under X-Windows which makes the program independent of the plat-

form on which it is used, provided the UNIX operating system is available. Detailed information on the hardware used and acquisition of the software can be obtained by writing to IPO-DLO.

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